THE ROLE OF NONCANONICAL NOTCH AND NUMB SIGNALING DURING HEART DEVELOPMENT

by
Edrick Sulistio

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Abstract

Heart development is a complex process with several signaling pathways intertwining to ultimately giving rise to a functional heart. There are still many aspects of heart development that are not fully understood. Canonical Notch signaling plays an important role in regulating early cardiac development when Notch intercellular domain (NICD) is cleaved at the membrane and translocated to the nucleus to activate transcription through the transcription factor RBPJκ. However, recent evidence suggests that Notch can also signal independent of RBPJκ, referred to as noncanonical Notch signaling. In this thesis, I aimed to investigate noncanonical Notch signaling during early heart development. To do this, I deleted RBPJκ and conditionally overexpressed Notch in mouse embryos using the cre-lox system and analyzed the developing embryos. Notch overexpression, independent of RBPJκ, resulted in the enlargement of the embryonic hearts due to increased myocyte proliferation. I developed two in vitro models by transfecting myocytes with constructs that overexpress either NICD or Tethered Notch. Tethered Notch is a membrane-bound Notch that cannot be cleaved to generate cytosolic NICD and thus cannot activate the canonical Notch signaling. Both in vitro models also exhibited increased cell proliferation as seen in in vivo model. Our lab recently demonstrated that the cytosolic protein Numb interacts with Notch to regulate noncanonical Notch signaling in stem cells. To analyze whether Numb plays a role in noncanonical Notch signaling during heart development, I transfected myocytes with constructs that knockout Numb and overexpress Numb to analyze Numb's effect on proliferation. Both knocking-out and overexpressing Numb increased cell proliferation. To conclude, both RBPJκ-independent Notch signaling and Numb mediate cardiac development, by regulating cell proliferation rate in myocytes.

Though their interaction in this context remains elusive, establishing a more definitive link between RBPJκ-independent Notch signaling and Numb in proliferation regulation will pave novel approach in studying cancer biology in other organs.

Advisors: Chulan Kwon, Ph.D. and Peter Andersen, Ph.D.

Preface

This thesis is the product of my Masters research initiated in August 2016 and finished in April 2017. The research was done in the Johns Hopkins University in Baltimore, Maryland.

I was first introduced to this project as my Master's research topic by Dr. Peter Andersen and Dr. Chulan Kwon. After learning canonical Notch signaling in class, I was intrigued in doing research on noncanonical Notch signaling. Furthermore, the enlarged heart phenotype that was observed really piqued my interest. As such, I decided to take on this project.

This thesis focuses on two different aspects: noncanonical Notch and Numb. The first half was focused on investigating the reason why the enlarge phenotype was seen. The latter half was relating the results obtained in the first half to Numb. This was done as an attempt to figure out the mechanism behind noncanonical Notch signaling.

I would like to this opportunity to thank Dr. Chulan Kwon, whom have provided me with the funding and guidance to do my study. I would also like to thank Dr. Peter Andersen for the extremely helpful discussion and mentorship he has provided me throughout the study. Throughout my research, I was fortunate enough to get help from several experts in and outside the laboratory. These people include: Amir Saberi, Sean Murphy, Emmanouil Tampakakis, Dr. Kathryn Tifft, Dr. Robert Horner, Matthew Miyamoto, Hye Kyung Shin, and Emma Zeng.

Finally, I would like to thank my family and friends for their continuous support throughout my research.

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Introduction

The heart is one of the most important organs in the human body; a minor defect or injury can be fatal. Cardiac malformation is present in one-percent of live births[1]. Despite progress in the treatment of cardiovascular disease, regenerating cells that were lost due to insults remains elusive. Hence, the study of heart regeneration is very important. For example, regenerating cells can replace scarred heart tissues. A good model for mammalian heart regeneration does not exist, so the focus of regenerative study has been cardiogenesis, heart development from a colony of pluripotent mesodermal stem cells.

Cardiogenesis starts when the embryo reaches gastrulation. After the formation of three germ layers (ectoderm, mesoderm, and endoderm), some of the mesodermal cells form cardiac crescent; the microenvironment within this cardiac crescent further restricts the mesodermal cell's destiny to cardiac lineage as promyocardial cells[2]. The restricted mesodermal cells contain distinct fields of cells that will eventually migrate from the cardiac crescent: first heart field (FHF) and secondary heart field (SHF) [2-4]. FHF cells will eventually form the primitive heart tube and provide a scaffold for SHF cells to migrate on[4,5]. SHF will develop into cardiac chambers – outflow tract, right ventricle, and atria, while FHF will develop into the left ventricle[6]. Though FHF and SHF cells originate from the mesodermal cells, these two fields differentiate at a different time in development[6]. FHF will first differentiate at the cardiac crescent stage, while SHF differentiates later upon migrating into the scaffold[4]. This variation in timing if

differentiation is partly due to the activation of a homeodomain protein interacting with complex feed-forward and inhibition signaling networks[7].

The first homeodomain protein expressed in mesodermal cells is *Mesp1*, which is transiently expressed in the mesodermal layer of the three germ layers, and marks the transition of the mesodermal cells into early cardiovascular progenitors[8]. Following *Mesp1* activation, *Isl1* is activated and promotes the SHF-progenitor cells to populate the heart[6]. *Isl1* activation is regulated through Fibroblast Growth Factor 10 transcription, though this regulation is only confirmed in some models such as human and mice[9]. As the cells differentiate, *Isl1* expression decreases while *Nkx2.5* expression increases[10]. When *Nkx2.5* is activated, the cells become more restricted to follow the cardiac lineage and thus, the *Nkx2.5*+ cells are called cardiac progenitor cells (CPCs)[10]. In vivo images showed that mesodermal cells that were previously $Isl1^+$ and expressed *Nkx2.5* are localized in the heart[10]. In vitro, $Nkx2.5^+$ cells can differentiate to cardiomyocytes, smooth muscle cells, and endothelial cells[11,12].

Mesp1, *Isl1*, and *Nkx2.5* proteins regulate complex signaling networks and are also regulated by those signaling networks. Wnt signaling, through β-catenin, is a critical regulator of the $Nkx2.5^+$ and $Isl1^+$ CPCs [13-15]. Recent data has shown that Notch signaling regulates Wnt signaling by directly negatively regulating β-catenin accumulation[16,17]. Notch signaling is canonically activated by the extracellular ligand Delta. Once Delta binds to the receptor, the activated receptor activates γ-secretase, which then cleaves the Notch Intracellular Domain (NICD). NICD is translocated into the nucleus and binds

RBPJκ/CSL, a transcription factor, to activate the target genes. The half-life of NICD in the cytosol is regulated by the Numb protein family which promotes NICD lysosomal degradation[16]. Thus, canonical Notch signaling is inhibited by Numb. However, Notch-dependent regulation of β-catenin is independent of ligand-dependent membrane cleavage of Notch and requires Numb, suggesting that Numb is able to regulate noncanonical Notch signaling[16].

Noncanonical can mean anything that deviates from canonical signaling pathway. Here, we define noncanonical Notch signaling as Notch signaling that can confer its effects without binding to its transcription factor (RBPJκ independent). This definition is based on the following results: (1) Conserved transcription factor-independent Notch signaling in *Drosophila*[18,19]; (2) Notch1's effect on myoblast differentiation into muscle cells independent of RBPJκ[20-22]; and (3) transcription factor-independent Notch signaling in other mammalian cells, specifically epithelial cell[23-25]. Thus, we believe that noncanonical Notch signaling is RBPJκ-independent, and have a strong role in development.

Conditional overexpression of NICD in mouse embryos results in an enlarged heart at embryonic day 9.5 (Kwon, unpublished data). I knocked-out RBPJκ in the same in vivo model to determine whether the phenotype is a result of increased canonical Notch signaling or noncanonical Notch signaling.

I hypothesized that overexpression of noncanonical Notch signaling will cause the enlargement of the heart. There are two reasons why an organ size can increase: increased cellular proliferation or increased cell size within that organ. Thus, I sought to explain the reason behind the enlarged heart phenotype. I also hypothesized that NICD interacts with Numb to mediate its noncanonical effects in the heart.

Methods

Pluripotent Stem Cells (PSCs) Culture and Cardiac Differentiation

Mouse PSCs with Numb floxed allele (Nb fl/fl, Nbl del/del, tom/tom) were maintained in 2i medium (Glasgow minimum essential medium with 10% fetal bovine serum, 1,000 U/ml ESGRO [Millipore], 3 μM CHIR99021, 1 μM PD0325901, Glutamax, sodium pyruvate, and MEM non-essential amino acids)[26]. For differentiation, cells were suspended in serum-free differentiation medium (SFD) (Iscove's modified Dulbecco's medium and F-12 medium, supplemented with B27, N2, Glutamax, ascorbic acid, and 1-thyolglycerol) for two days[27]. Cells were treated with Activin A, bone morphogenetic protein 4 (BMP4), and vascular endothelial growth factor (VEGF) in SFD medium for two days. The media was replaced with fresh SFD medium (without the cytokines) once every two days. On day 7, the myocytes were dissociated using TrypleE Express (Life Technologies, Cat. #12604013) and plated on a 24-well plate for transfection.

Plasmid Constructs and Transfection Protocol

The following plasmids were used: pCAG-cre-IRES-Puro, pCAG-Tethered Notch-GFP (pCAG-TN-GFP), pCAG-GFP-IRES-PURO, pCAG-Notch Intracellular Domain-GFP (pCAG-NICD-GFP), and pCAG-Numb-GFP-IRES-Puro[28]. Transfection was carried out with Lipofectamine® 3000 (Life Technologies) on Day 7 of differentiating cell line. Protocol presented in the product was followed.

Immunohistochemistry, Flow Cytometry Staining, Imaging, and Analysis

Six days post-transfection, the cells to be imaged were fixed with 4% paraformaldehyde for 20 minutes. The cells were permeabilized with permeabilization solution (1% BSA, 1% FBS, 0.2% Triton in PBS) for 5 minutes. The following primary antibodies were introduced to permeabilized cells and incubated overnight: 5F8 anti-red (ChromoTek, Cat. # 120328), GFP Tag Polyclonal Antibody (ThermoFisher Scientific, Cat. # A10262), Troponin T Cardiac Isoform Ab-1 Mouse monoclonal Antibody (ThermoFisher Scientific, Cat. #MS-295-P1), and Anti-Phospho-Histone H3 (Ser10) Mitosis Marker (EMD Millipore, Cat. #06-570). After overnight incubation of the primary antibodies, the following secondary antibodies were introduced and incubated for 1 hour: donkey anti-rat IgG Alexa Fluor® 594 (ThermoFisher Scientific, Cat. #A-21209), donkey anti-mouse IgG Alexa Fluor® 647 (ThermoFisher Scientific, Cat. #A-31571), donkey anti-mouse IgG Alexa Fluor® 594 (ThermoFisher Scientific, Cat. #A-21203), goat anti-chicken IgY Alexa Fluor® 488 (ThermoFisher Scientific, Cat. #A-11039), donkey anti-rabbit IgG Alexa Fluor® 647 (ThermoFisher Scientific, Cat. #A-31573), and donkey anti-mouse IgG Alexa Fluor® 488 (ThermoFisher Scientific, Cat. #A-21202). DAPI (ThermoFisher

Scientific, Cat. #D1306) staining was performed for 5 minutes. In PBS, cells were imaged with EVOS FL Cell Imaging System (ThermoFisher Scientific). For flow cytometry staining, donkey anti-rat IgG Alexa Fluor® 594 was replaced with goat anti-rat IgG PE conjugate (Life Technologies, Cat. #PA1-29628), and no DAPI staining was introduced. Cells were analyzed by flow cytometry as previously described[28].

Mice Maintenance, Embryonic Harvest, Staining, and Sectioning

Nkx2.5-cre, Notch overexpressed, and RBPJκ fl/fl (JAX Lab) mice were used. *Nkx2.5*-cre mice were mated to RBPJκ fl/fl, yielding the new line of mice with *Nkx2.5*-cre and RBPJκ fl/+. These new mice are then mated with Notch overexpressed, RBPJκ fl/fl mice to yield the embryos with the desired genotypes. The mice were taken care of in the Johns Hopkins University Animal Care and Use Facility under protocol Number MO10M444. To obtain mouse embryos, the Notch overexpressed and RBPJκ fl/fl mice (mated with *Nkx2.5*-cre and RBPJκ fl/f+ mouse) were pregnant for at least 8 to 9 days before dissection. Fertilized female mice were confirmed by checking their vaginal plug. The embryos were harvested, fixed, sectioned, stained, and imaged as previously described[28]. The antibodies used were previously mentioned above, in addition to Wheat Germ Agglutinin Alexa Fluor® 647 Conjugate (ThermoFisher Scientific, Cat. #W32466), Islet-1 and Islet-2 homeobox rat antibody (Developmental Studies Hybridoma Bank, Cat. #39.4d5), and Nkx-2.5 mouse monoclonal IgG_1 (Santa Cruz Biotechnology, Cat. #sc-376565).

Results

Overexpression of Notch Intracellular Domain, Independent of Transcription Factor RBPJκ, Increases Heart Size in Embryonic Day (E) 8.5 and 9.5 Mice

My laboratory proposed the existence of noncanonical Notch signaling. They discovered that the overexpressing Notch Intracellular Domain (NICD) causes the enlargement of the heart at E9.5 (Kwon, unpublished data). If the heart enlargement phenotype is also observed in embryos with a complete knock-out of RBPJκ, we can show that Notch signaling in this system can occur independently of its transcription factor. To control temporally the knocking-out of RBPJκ, we express cre under the control of *Nkx2.5* promoter as this promoter is activated at E7.5.

To get *Nkx2.5*-cre, RBPJκ flox/flox, complete Notch Overexpression (OE)/+ embryos, I crossed *Nkx2.5*-cre, RBPJκ fl/+ mice with Notch OE/OE, RBPJκ fl/fl mice. I genotyped the embryos based on the tail or yolk sac and confirmed their genotype (agarose gel not shown). Embryos that were negative for cre, did not have Notch OE and loss of RBPJκ, and thus, conferred a wild-type phenotype; these embryos were used as the control. To calculate the length of the heart, I measured the images in Figure 1 directly using inches. In control E8.0 embryos, the average heart length was 0.26 inches (Figure 1A). In contrast, the average length of mutant E8.0 hearts was 0.39 inches (Figure 1B). The average length of the mutant embryonic hearts at E9.0 was 1.36 inches (Figure 1F), while the average length of the control heart of the same age was 1 inch (Figure 1D). In E9.5, the average length of the control heart is 1 inch (Figure 1H) and 1.2 inches for mutant heart (Figure 1J). In summary, the hearts of cre-positive embryos (mutant) (Figure 1B, F, and J) were larger than the hearts of the control embryos (Figure 1A, D, and H).

There were two embryos with severe phenotype at age E9.0 and E9.5 (not shown). The E9.0 embryo had a retarded growth while E9.5 embryo had a much smaller body size. These two phenotypes will be discussed in the next section.

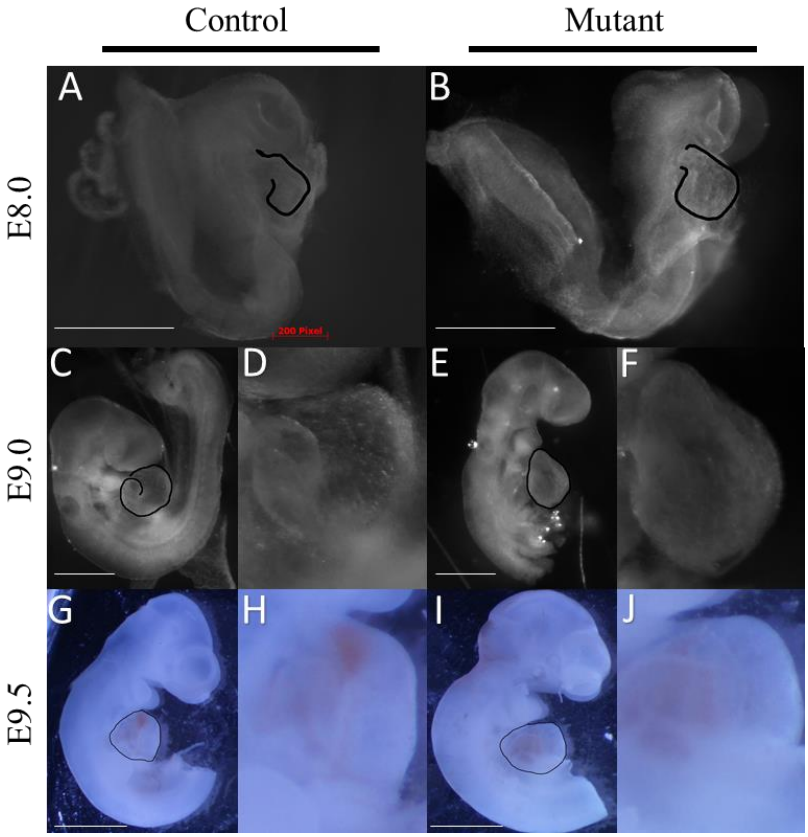

Figure 1 Noncanonical Notch signaling overexpression caused heart enlargement in E8.0, E9.0, and E9.5. Representative images of embryos and their hearts at embryonic day (E) 8.0, 9.0, and 9.5. The hearts (A-C, E, G, and I) are outlined with black. C, E, G, and I's images are enlarged in D, F, H, and J, respectively, to see the heart. White bar represents 500 micrometer.

Overexpression of Notch Intracellular Domain, In the Absence of RBPJκ, Increases In vivo Myocyte Proliferation Rate

To test whether the enlargement of the heart is due to increased proliferation rate or cell size, I sectioned and stained the embryos with Wheat Germ Agglutinin (WGA) and Phospho-Histone H3 (PH3). WGA binds to the plasma membrane of the cells, which allows the visualization of the cell size. To label proliferating cells, we detected

phosphorylated histone H3 in the nucleus. Histone H3 is known to be phosphorylated during both mitosis and meiosis. Thus, detecting phosphorylated H3 is an excellent way to quantify the number of cells undergoing proliferation. We also stained for Nkx2.5, a marker for myocytes (Figure 2 B, G, L, and Q) to inform us which cells are myocytes. Simply asking why the heart is enlarged is not sufficient to understand the process; we must also answer when those cells become larger or proliferate more.

I discovered that there was a higher number of proliferating cells in mutant heart compared to control heart (Figure 2A-T). In addition, most of the proliferating cells were positive for Nkx2.5 antibody, suggesting that they were all myocytes (Figure 2 E, J, O, and T). There were higher number of proliferating cells in the niche compared to heart (Figure 2A-T). Niche is defined as the tissue wherein cardiac progenitor cells migrate from. As such, Nkx2.5-positive cells are rarely seen in this tissue, as observed (Figure 2A-J).

I looked at cell size by staining the embryonic hearts with WGA (Figure 2 U and W). Here, I only compared the myocyte (Figure 2 V and X) size because of the preferred proliferation rate increment in myocytes (Figure 2A-T). I measured the maximum length of all the cells shown in the images (Figure 2U-X) and found that there was no significant myocyte size difference between the control and mutant (Figure 2Y).

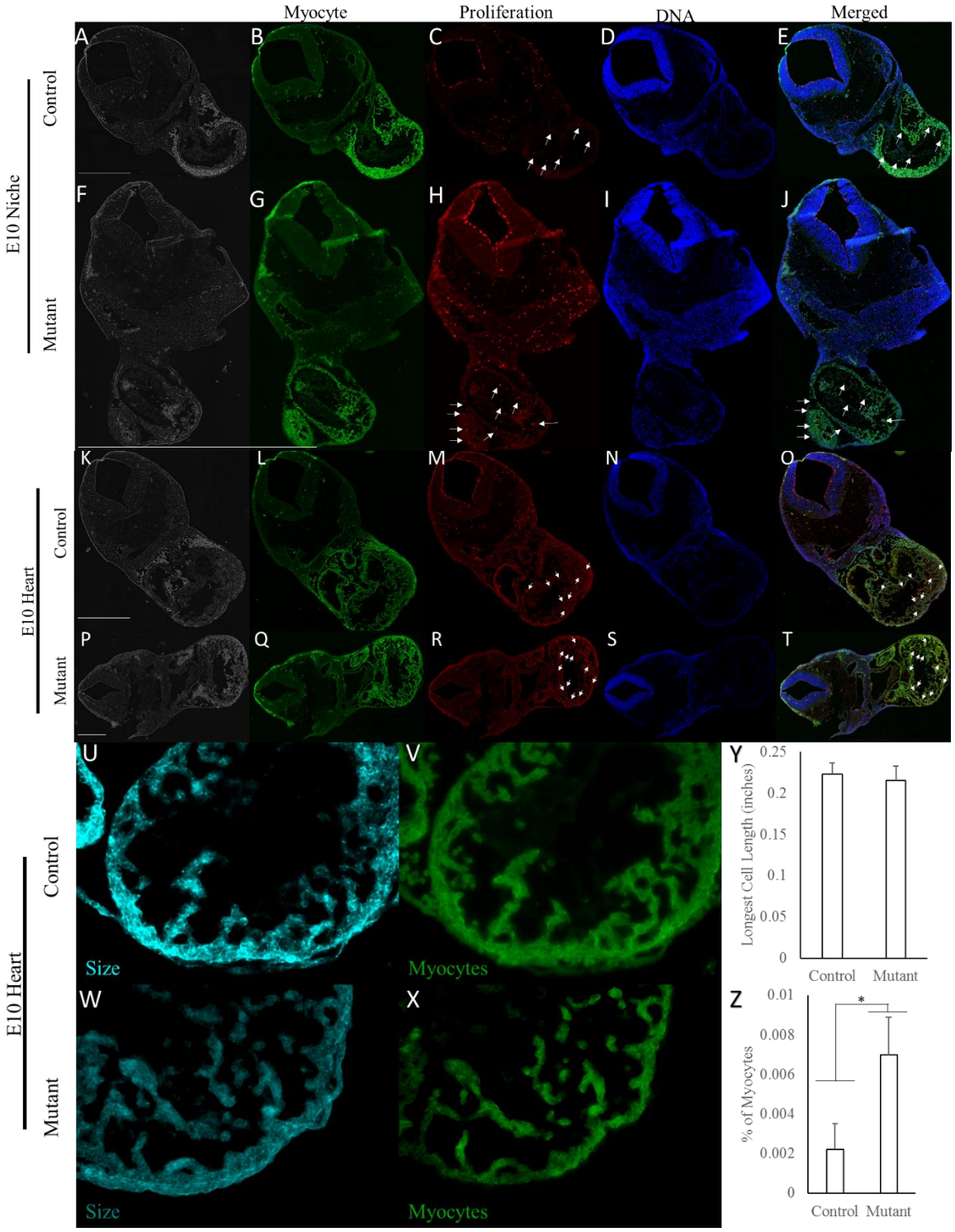
Myocyte
Proliferation
DNA
Merged
E10 Niche
Control
Mutant
E10 Heart
Control
Mutant
E10 Heart
Control
Mutant
Size
Myocytes
Size
Myocytes
Longest Cell Length (inches)
0.25
0.2
0.15
0.1
0.05
0
Control
Mutant
% of Myocytes
0.01
0.008
0.006
0.004
0.002
0
*
Control
Mutant

Figure 2 Noncanonical Notch overexpression increases myocyte proliferation rate. Representative images of sectioned embryonic niches (A-J) and hearts (I-X) at E10. Sections (A, F, K, and P) were stained with Nkx2.5 for myocytes (B, G, L, and Q), PH3 (C, H, M, and R), and DAPI (D, I, N, and S). Merged images are shown (E, J, O, and T). Embryonic hearts were also stained with WGA (U and W) and Nkx2.5 (V and X). White arrows show proliferating cells. Scale bar is provided as the white bar, indicating 500 micrometer. (Y) shows the comparison of maximum cell length between control and mutant ($p>0.05$). (Z) shows the comparison of myocyte percentages found in embryonic heart between control and mutant. * indicates $p<0.05$.

To show the difference of myocyte proliferation rate in control and mutant embryos, we isolated the embryonic hearts (both mutant and control) and calculated the percentages of myocytes within the heart. We confirmed that there is higher number of myocytes in mutant embryo compared to control (Figure 2Z) (Kwon, unpublished data).

Overexpression of Tethered Notch (TN) and NICD In vitro Increases Cell Proliferation Rate

I wanted to test if my in vitro model, Numb floxed (Nb fl/fl, Nbl del/del, tom/tom) cell line, simulates the increased cell proliferation phenotype found in vivo. Briefly, this cell line has deleted Numblike (Nbl) genes and the ability to express RFP (tomato/tomato) when cre recombinase is introduced, signifying the knockout of Numb genes (Figure 3). I chose this cell line because I believed that Numb is a candidate protein within the noncanonical Notch signaling pathway due to its role in the regulation of β-catenin through noncanonical Notch signaling. I transiently transfected this Nb fl/fl, Nbl del/del, tom/tom cell line with pCAG-TN-GFP and pCAG-NICD-GFP on day 7 of differentiation. Afterwards, I fixed and stained transfected cells with cardiac troponin T (cTNT) (to mark which cells are myocytes), Phospho-Histone 3 (PH3), and GFP to amplify the internal GFP signal. Briefly, Tethered Notch (TN) is a membrane-bound Notch that cannot be cleaved by *γ-secretase*. As such, it cannot be utilized for canonical

Notch signaling. On the other hand, Notch Intracellular Domain (NICD) is the cleaved intracellular domain of the Notch receptor. This protein can still translocate into the nucleus and bind to RBPJκ. However, overexpression of NICD means that some of the NICD will not be used for canonical signaling. As such, whatever remains of the NICD can be utilized for noncanonical signaling. Preliminary study through visual observation to compare and contrast the number of proliferating cells within transfected and non-transfected cell population was performed first through manually calculating the number of transfected cells (Figure 4A-H) and whether they proliferate much more compared to non-transfected cells (Table 1).

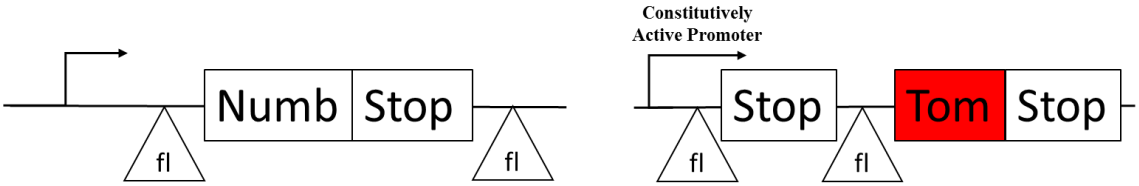

Figure 3 Schematic diagram of the Numb fl/fl, Nbl -/-, and tom/tom cell line. Nbl is not shown because it is deleted. Introduction of cre will knockout the genes between the fl sites (triangles). Tom is a RFP (shown in red box).

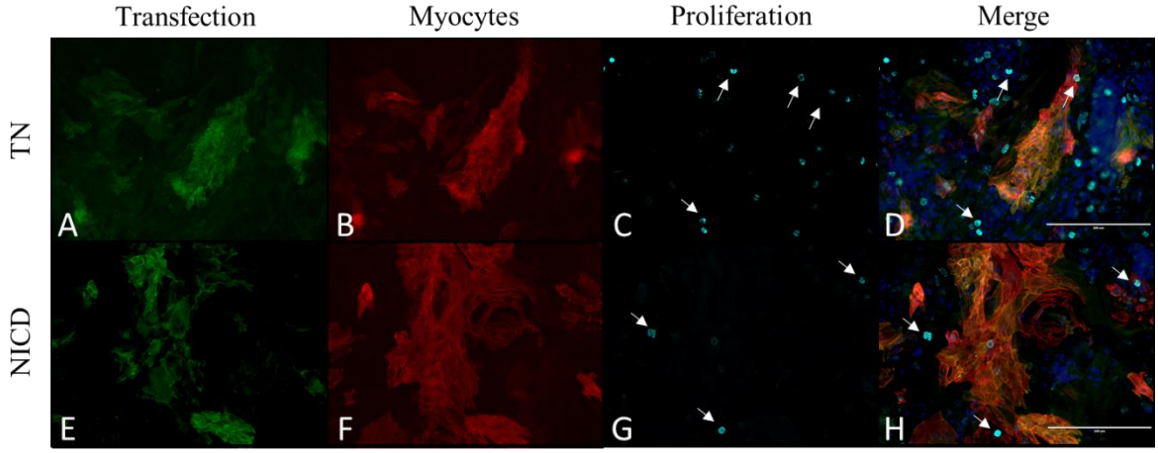

Figure 4 Representative images of cells overexpressing TN and NICD. Cells transiently expressing (A) Tethered Notch-GFP, stained with (B) cTnT, (C) PH3. Cells transiently expressing (E) NICD-GFP, stained with (F) cTnT, (G) PH3. The merged images (D and H) are shown. White arrow represents phosphorylated histone H3. White bar represents 200 micrometer.

Group	Construct	Percentage (%)
Internal Control	None (n = 11)	16.67
Experimental	TN (n = 55)	83.33
Internal Control	None (n = 20)	29.41
Experimental	NICD (n = 48)	70.59

Table 1 Percentage of proliferating cells in transfected and non-transfected cell population. TN is Tethered Notch while NICD is Notch Intracellular Domain. Cell count was measured through visual observation.

This data suggests that the overexpression of TN and NICD increased cell proliferation. The weakness of this data was the potential for bias during data analysis. In addition, the standard for a signal to count as positive I utilized between cells may varied. To confirm this data and eliminate bias, I analyzed the signals by Flow Cytometry, which selected for cells with signals under the same standard throughout the experiments.

FACS analysis confirmed that TN-transfected cells are proliferating more compared to non-transfected cells based on PH3 staining. Out of the 61.7% of transfected cells, 0.99% were actively proliferating or about to enter mitosis (Figure 5A). This means 1.6% of cells within TN-transfected cell population were proliferating. Meanwhile, the control population of cells only had about 0.5% of proliferating cells. Utilizing this calculation, after repeating the experiment for four times, the average percentages of proliferating cells in TN-transfected cells and non-transfected cells are 2.85%, and 1.03%, respectively (Figure 5C). Thus, the proliferation rate (percentage of proliferating cells in TN-transfected cells divided by percentage of proliferating cells in non-transfected cells) of TN overexpressing cells was magnified 2.76 times-fold compared to control. In NICD transfected cells (Figure 5 B and D), using the same method of calculation as in TN-transfected cells, I found that the proliferation rate was magnified by 3.88 times-fold

compared to non-transfected cells. This data showed two things: (1) overexpression of both TN and NICD increased cell proliferation, as shown by the in vivo data, and (2) the Numb fl/fl, Nbl del/del, tom/tom cell line can be used to investigate the downstream pathway of noncanonical Notch signaling. Myocytes were also transfected with pCAG-GFP-IRES-Puro as a control. The proliferation rate between GFP-transfected myocytes and non-transfected myocytes had no significant differences (data not shown).

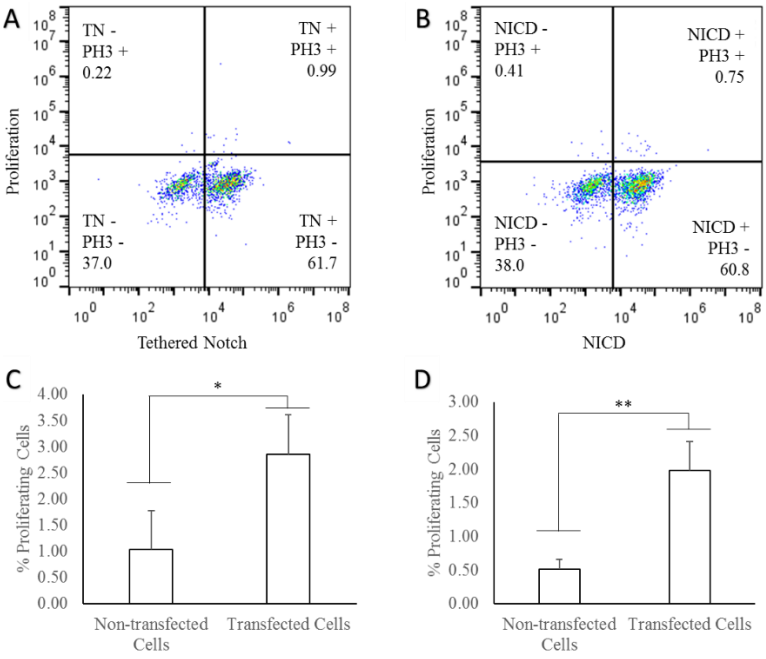

Figure 5 NICD and TN overexpression increases myocyte proliferation rate. Tethered-Notch transfected cells are plotted against PH3 signal strength (A). NICD transfected cells are plotted against PH3 signal strength (B). The average percentage (n=4) of proliferating cells within the respective non-transfected and transfected cells are shown (C and D). * and ** represent p values < 0.05 and < 0.005, respectively.

Numb Double-Knockout (DKO) and Overexpression Increase Proliferation Rate In vitro

After showing that the overexpression of noncanonical Notch signaling promotes cell proliferation, I sought to understand how noncanonical Notch signaling regulates cell proliferation. We hypothesized that Numb might play a role in noncanonical Notch signaling. Numb is an endocytic adaptor protein with varying functions within the cell ranging from control of proliferation to degradation of protein [16,29-31]. In mice, there are many isoforms of Numb. The most prominent isoforms, Numb (Nb) and Numb-like (Nbl) proteins are functionally redundant [32]. Therefore, both genes must be knocked-out to observe a phenotype. The heart size in mouse embryos at E13 was increased in Numb

double knockouts (cre+, Nb fl/fl, Nbl fl/fl) [33]. This phenotype is very similar to the phenotype of NICD overexpression (shown above). In addition, the noncanonical interaction between Numb and Notch to regulate β-catenin[16] further shows that Numb and NICD interacts in the cell. Thus, I hypothesized that the double knocking-out of Numb and Numb-like causes increased cell proliferation in myocytes. In addition, I also hypothesized that overexpressing Numb will decrease the proliferation rate of myocytes.

Before I started my experiment, I confirmed that transfection of cre (pCAG-ilsCre-IRIS-Puro) to Numb floxed allele cell line (Nb fl/fl, Nbl del/del, tom/tom) caused Numb deletion (data not shown). Afterwards, I transfected cre in Numb floxed allele cell line (Nb fl/fl, Nbl del/del, tom/tom) on Day 7 of differentiating cells (Figure 6A-E). When cre was transfected, I observed an increased cell proliferation rate in transfected cells. Utilizing FACS analysis, I found that 0.22% of the cell population were cre transfected and proliferating cells, 0.86% were proliferating non-transfected cells, 10.5% were non-proliferating cre-transfected cells, and 88.4% were non-proliferating non-transfected cells (Figure 7A). The percentage of proliferating cre-transfected cells compared to non-proliferating but transfected cells is 2.09%, while the percentage of proliferating non-transfected cells compared to non-proliferating non-transfected cells is 0.97%. After repeating the experiment five times, I found that the average percentage of proliferating cells in Numb double-knockout cells is 1.63%, in contrast with non-transfected cells' average percentage of proliferating cells 0.48% (Figure 7C). Thus, cre-transfected cells proliferate more by 3.39 times-fold compared to non-transfected cells.

To overexpress Numb, I transfected the same cell line (Nb fl/fl, Nbl del/del, tom/tom) with pCAG-Nb-GFP-IP (Figure 6F-J). Surprisingly, increased cell proliferation

was observed. Numb-overexpressed cells proliferate about 1.3 times-fold more than the control (ratio of transfected cell: 0.0083, ratio of non-transfected cell: 0.0053) (Figure 7B and D).

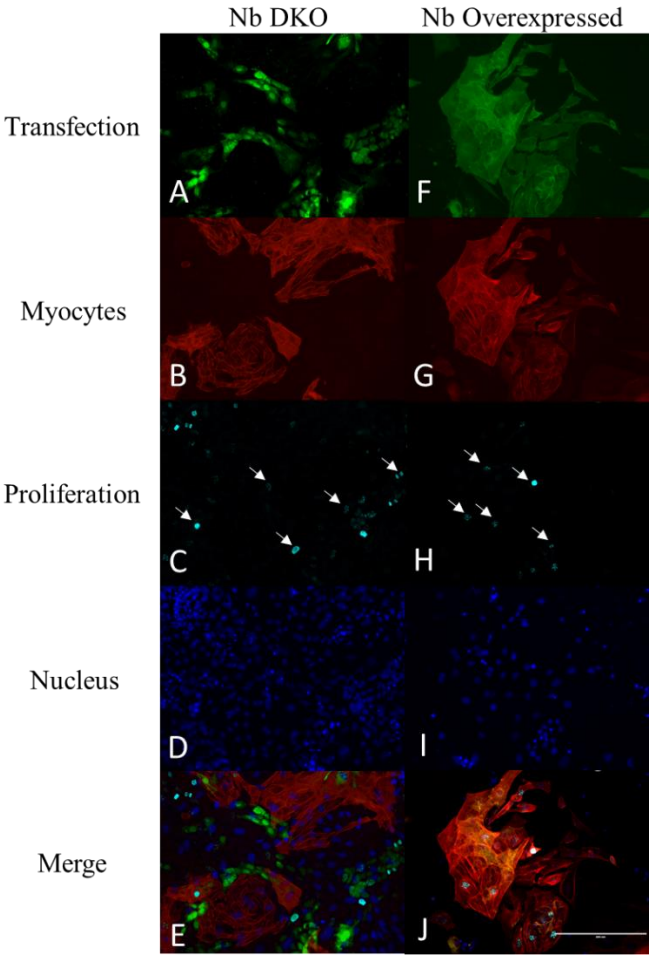

Figure 6 Representative images of cardiomyocytes transfected with cre and Numb. Cells were transfected with constitutively active cre (A) and Nb (F), stained with (B and G) cTnT, (C and H) PH3, and (D and I) DAPI. (E and J) show the merge of the above images. White arrow shows the phosphorylated histone H3, marking only cells that are in the process of proliferating. White bar represents 200 micrometer.

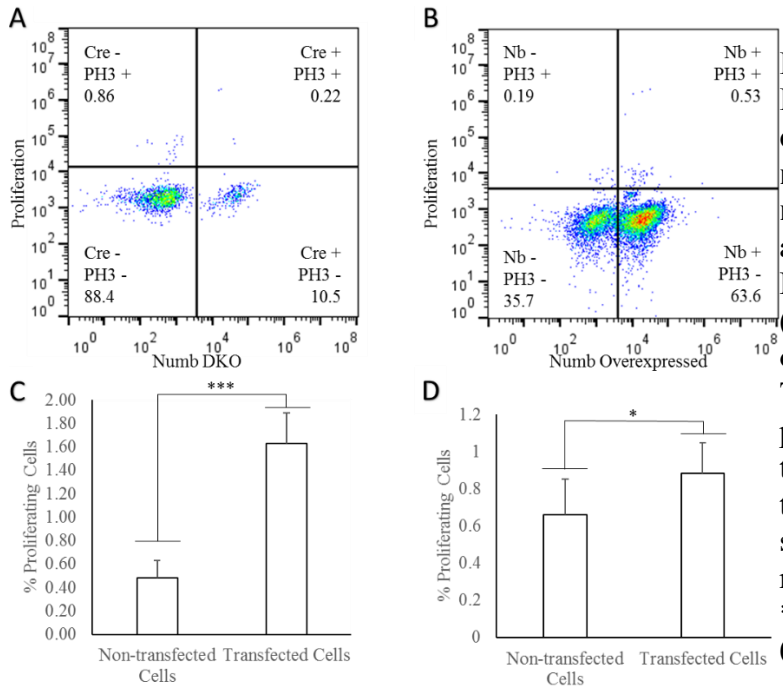

Figure 7 Numb Double-Knockout (DKO) and overexpression increases myocyte proliferation rate. Flow cytometry analysis of (A) Numb Double Knockout (DKO) (n=5) and (B) Numb overexpression (n=3). The mean percentage of proliferating cells within the non-transfected and transfected cells are shown (C and D). * represents p value < 0.05. *** represents p value < 0.0005.

Numb Double Knock-out and Tethered Notch or NICD Overexpression Markedly Increases Cell Proliferation

In my hypothesized model, NICD promotes myocyte proliferation, while Numb should have inhibited myocyte proliferation. Thus, NICD and Numb act antagonistically (Figure 8). To test my hypothesis, I co-transfected the in vitro model with cre and TN or NICD constructs to yield a cell line with double-knockout Numb and overexpressed TN or NICD. According to my model, this co-transfection should result in increased myocyte proliferation. All the cells within the flow cytometry graph were myocytes. Within the cell population, 11.7% represent cre and NICD co-transfected myocytes, 54.9% represent NICD-only transfected myocytes, 7.10% represent cre-only transfected myocytes, while the remaining 26.3% represent non-transfected cells (Figure 9A). Within that 11.7% of cre and NICD co-transfected myocytes, 0.47% of cells were actively proliferating (Figure 9B). Meanwhile, only about 0.035% of non-transfected myocytes were proliferating (Figure 9C). In cre and TN transfection, 15.9% of myocytes were confirmed to be co-

transfected, 49.9% were TN-only transfected myocytes, 9.05% were cre-only transfected myocytes, and the remaining 25.1% did not get transfected at all (Figure 9D). Within 15.9% of TN and cre co-transfected myocytes, 0.41% of them were actively dividing (Figure 9E), in comparison to only 0.024% were actively dividing in non-transfected cells (Figure 9F).

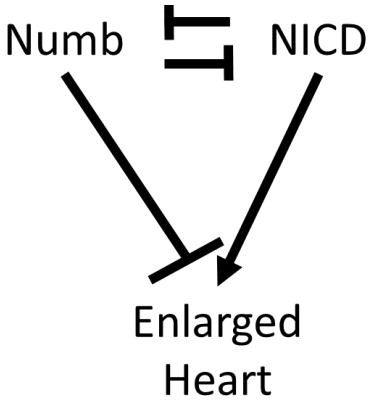

Figure 8 Proposed model of the interaction between NICD and Numb in noncanonical Notch signaling.

With the same method of calculation used to determine the percentage of proliferating cells in TN and NICD transfected cells, and after three trials, I found that the NICD and cre co-transfected cells proliferated 123-times fold more than the non-transfected cells (percentage of proliferating co-transfected cells: 5.28%, while ratio of non-transfected cells: 0.04%) (Figure 9G). A similar phenotype was also found in TN and cre co-transfected cells, with about 11-times fold more than non-transfected cells (percentage of proliferating co-transfected cells: 3.03%, while ratio of non-transfected cells: 0.27%) (Figure 9H).

Numb Overexpression and Tethered Notch or NICD Overexpression Increases Cell Proliferation

Based on the model I proposed earlier (Figure 8), overexpression of both Numb and TN or NICD should rescue the phenotype and normalize the proliferation rate to that

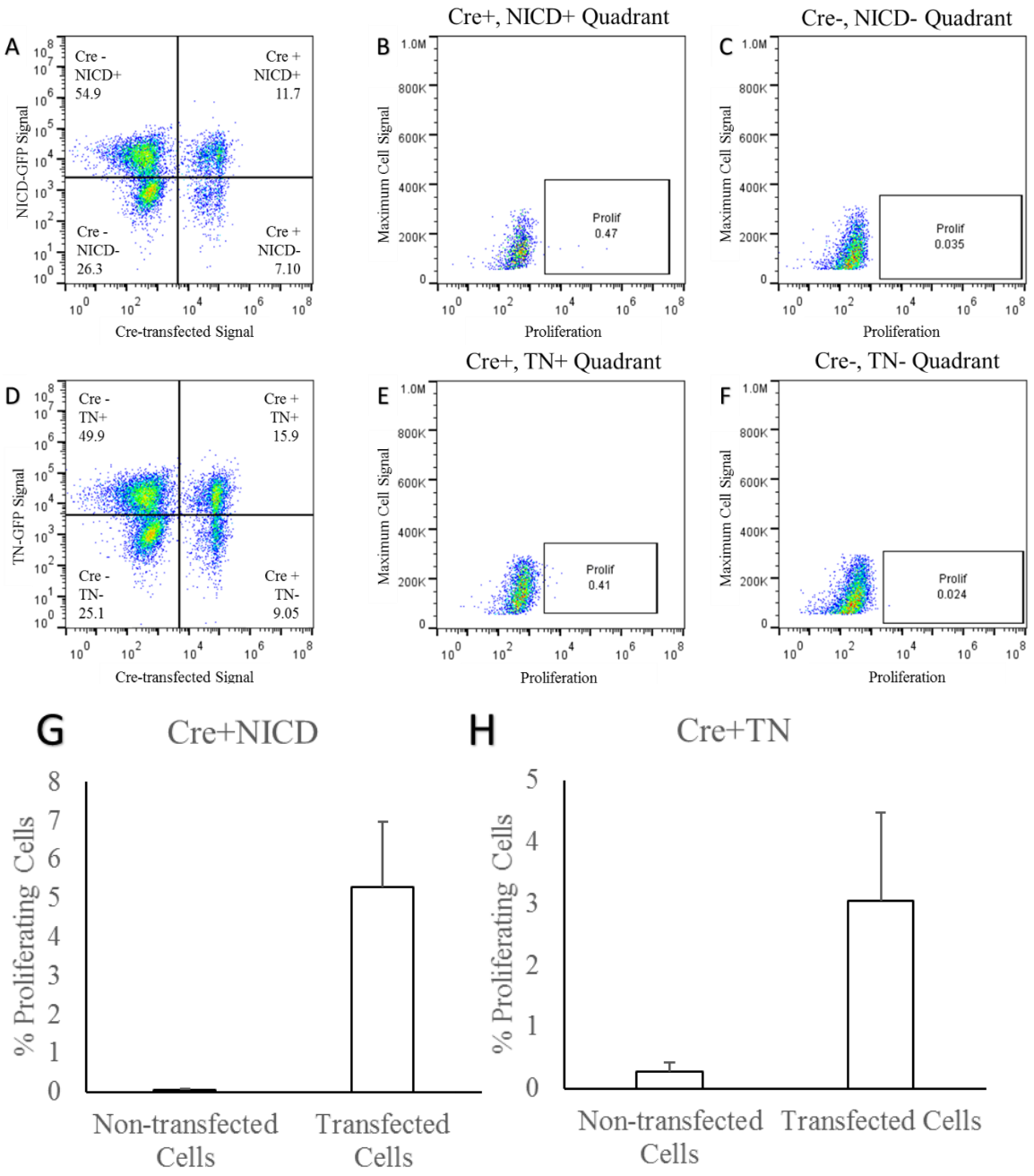

Figure 9 Myocytes with both double-knockout (DKO) Numb and overexpressed NICD/TN have higher increased proliferation rate. (A) shows the population percentage of Cre and NICD transfection, (B) shows the percentage of cells proliferating within Cre and NICD co-transfected cells, (C) shows the percentage of cells proliferating in control, non-transfected cells, (D) shows the population percentage of Cre and TN transfection, (E) shows the percentage of cells proliferating co-transfected cell population, and (F) shows the percentage of cells proliferating in control, non-transfected cells. The average percentage (n=3) of proliferating cells (compared to non-transfected cells) are shown (G and H).

of non-transfected cells. To test my hypothesis, I co-transfected the in vitro model with Numb and TN or NICD.

In NICD and Numb co-transfected cells, I found that the percentage of proliferating cells is 6.02%, in contrast with the non-transfected cells 0% (Figure 10A). After performing two trials, the average percentage of proliferating cells in NICD and Numb co-transfected cells is 3.32%, in contrast with the average percentage of proliferating cells in non-transfected cells 1.80% (Figure 10C). Thus, the NICD and Numb co-transfected cells proliferated 1.84-times fold more than non-transfected cells.

This increased proliferation was also observed in TN and Numb co-transfected cells. The percentage of proliferating co-transfected cells is 14%, while the percentage of proliferating non-transfected cells is 0.16% (Figure 10B). After performing three trials, the average percentage of proliferating cells in TN and Numb co-transfected cells and non-transfected cells are 2.08% and 0.53%, respectively (Figure 10D); TN and Numb co-transfected cells proliferated 3.86-times fold more than non-transfected cells.

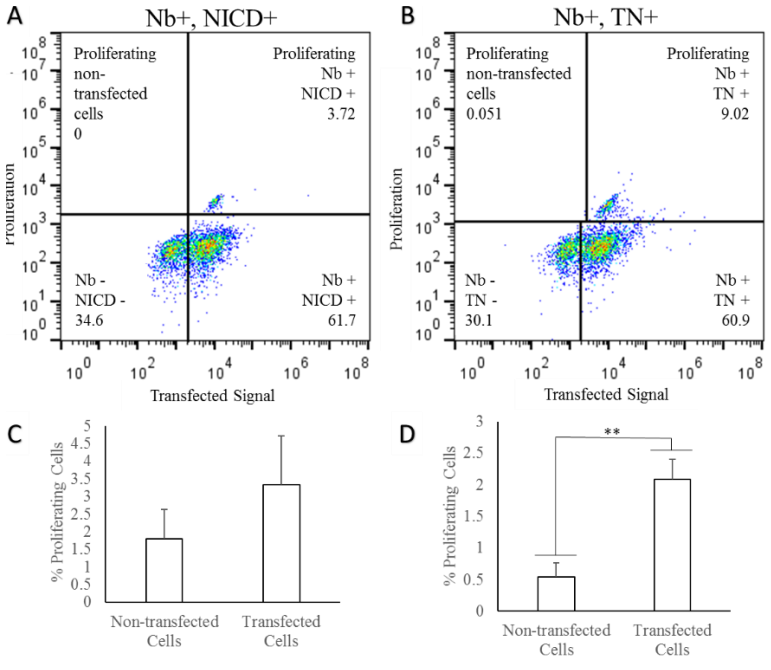

Figure 10 Numb and NICD/TN co-transfected myocytes also experienced increased proliferation rate. Flow cytometry analysis of (A) Numb and NICD overexpression (n=2) and (B) Numb and TN overexpression (n=3). The mean percentage of proliferating cells within the non-transfected and transfected cells are shown (C and D). ** represents p value < 0.005.

Discussion

In this study, we confirmed the existence of noncanonical Notch signaling in late cardiac development. By overexpressing Notch when *Nkx2.5*, an important homeodomain for cardiac progenitor cells differentiation, is activated, I discovered that the embryonic hearts at E8.0, E9.0 and E9.5 are enlarged. Our data suggests that the increased proliferation rate is the primary cause of the enlarged phenotype. Cardiac progenitor cells are still able to proliferate as they migrate to form FHF and SHF cells. One of the questions we tried to answer was the temporal regulation behind this proliferation: did the increased proliferation occur in cardiac progenitor cells? Or did it occur once the cells have become myocytes? The in vivo results suggested that increased proliferation occurred in myocytes due to positive PH3 signals only in cells stained with Nkx2.5, in addition to the increased number of myocytes compared to control.

Surprisingly, I found one E9.0 embryo with retarded growth (appearance similar to E8.5, instead) and E9.5 embryo with a significantly smaller body size (data not shown). Upon more investigation, the E9.0 embryo did not express any cre at all and thus, may just experience random mutation within its system to cause slower growth. As for the E9.5 embryo case, the smaller body phenotype may be caused by lack of circulation to the body from the embryo's heart. The increased heart size may block the arteries going out of the heart and/or the vein going into the heart. As such, the embryo lacked all the necessary nutrition to grow, causing it to have a stunted growth.

Next, I attempted to prove that my in vitro model, when overexpressing Notch, can mimic the increased cell proliferation phenotype found in the embryos. I transfected the differentiating Nb fl/fl, Nbl del/del, tom/tom cell line with the constitutively-expressing Tethered-Notch (TN) construct or Notch Intracellular Domain (NICD). We found that there is a markedly increased proliferation rate in both transfections. Thus, we confirmed that we can utilize our in vitro model to simulate in vivo noncanonical Notch signaling. However, there is a slight difference in proliferation rate between NICD-transfected myocytes and TN-transfected myocytes. Since the Nb fl/fl, Nbl del/del, tom/tom cell line has a normal Notch signaling, some of the expressed NICD might be utilized for canonical Notch signaling. This means that the remaining NICD would be available for noncanonical Notch signaling and/or degraded. Thus, it was expected that there is a lower proliferation rate in NICD-transfected myocytes compared to TN-transfected ones. However, our data suggests otherwise. I reasoned that better NICD transfection rate, in contrast to TN transfection rate, was the primary reason why NICD-transfected myocytes proliferated more.

After confirming that my in vitro model works, I sought to understand the possible role of Numb in noncanonical Notch signaling by utilizing Nb del/del, Nbl del/del, tom/tom cell line. Unfortunately, for an unknown reason, the stem cells failed to differentiate into myocytes, let alone cardiac progenitor cells (CPCs) (results not shown). As a result, I chose the Nb fl/fl, Nbl del/del, tom/tom cell line. As explained previously, this cell line contains the floxP site in the absence of cre, so Numb is expressed normally. Perhaps, in the future, engineering a new inducible cell line that can double-knockout

Numb should be done for better temporal control. The knocking-out of Numb in my in vitro system showed that the myocytes proliferate much faster. Interestingly, the amount of cre-transfected myocytes is very low. Initially, it was reasoned that transfected non-myocytes may have proliferated in a much faster rate than transfected-myocytes. This reason seemed plausible at first because I saw more non-myocytes growing. However, the analysis from the flow cytometry data ruled that reason out. Upon more investigation and better differentiation, I found that most of the beating myocytes failed to survive the transfection. The survival rate of cre-transfected cells in comparison to control cells (both GFP-only transfected cells and Lipofectamine-in-media cells) is much lower. I therefore hypothesized that the cre-transfected cells failed to attach to the gelatin-coated wells. The reason behind this is simply because Numb is an adaptor protein, and the double knockout of Numb and Numblike may have perturbed the cell's cell-matrix interaction. This hypothesis was confirmed to be possibly true as Numb double knockout decreases the expression level of β-integrin (Kwon, unpublished data).

I also increased Numb expression in vitro to see if I can find the opposite phenotype: decreased proliferation rate. Surprisingly, there was also an increase of cell proliferation rate, albeit lower than the Numb knockout's proliferation rate. Perhaps, the overexpression of Numb triggers a different pathway – Numb has been proven to interact with a lot of other proteins. Combining these results together, we therefore established a link between noncanonical Notch signaling and Numb. A relationship between noncanonical Notch signaling and Numb has been seen when Notch regulates β-catenin through Numb and lysosome in stem and progenitor cells[16]. As such, our results fit within

a framework established in earlier stage of development. To further strengthen the link between Numb and noncanonical Notch, a lot more experiments must be conducted. Here, we attempted experiments to simulate rescue or intensify phenotype of mutant. We co-transfected myocytes with cre or Numb and TN or NICD – this setup provided us with four different transfection experiments. As expected, the knocking-out of Numb and overexpression of TN or NICD further increases myocyte proliferation rate. However, the overexpression of Numb and overexpression of TN or NICD did not make the proliferation rate normal – instead, it further increases cell proliferation rate, consistent with our single transfection of Numb. Thus, I reason that in this Numb and TN or NICD co-transfection, other signaling pathways (and not non-canonical Notch signaling) must have been turned on which resulted in faster proliferation. Nevertheless, more experiments must be done as follow-up.

These results do not evaluate binding between Numb and noncanonical Notch signaling (through NICD). Perhaps, the experiment to show this binding can be done through co-immunoprecipitation to show direct/indirect binding between Numb and NICD. However, one possible problem with this co-IP experiment is the effect of canonical Notch interacting with Numb. As stated earlier, we already know that Numb binds to NICD to undergo lysosomal pathway for canonical Notch regulation in stem cells [16]. Thus, a new mouse model must be developed wherein we can alternatively overexpress noncanonical Notch signaling (and only through Tethered Notch) and double-knockout Numb and Numb-like to see if we can see a rescue phenotype. The rationale behind this suggestion is due to the fact that NICD and Numb are interacting

with each other. In my model, overexpressing noncanonical Notch signaling in addition to overexpressing Numb can normalize the proliferation rate of myocytes, though this contrasts with in vitro results (Figure 10). Thus, further study must be done on in vivo Numb double-knockout effect on mouse heart.

Admittedly, one weakness of my in vitro model is the transient expression of the TN and NICD without interrupting the pre-existing canonical Notch signaling that is occurring within the cell. However, within our in vivo model, we have seen that with and without knocking-out of RBPJκ/CSL, the phenotype of the heart remains the same, suggesting that the overexpression of Notch may have a greater impact noncanonically.

My results indicated that noncanonical Notch signaling has a greater role than regulating β-catenin level in mice cardiac development. Here, we showed that noncanonical Notch signaling also regulates the proliferation rate of myocytes. Therefore, more studies must be conducted on noncanonical Notch signaling across all systems and models to differentiate whether the observed phenotype was due to canonical or noncanonical Notch signaling. Solving this mystery can provide us with a better understanding on how noncanonical Notch signaling affects development, which can then be translated to human studies. Perhaps, some congenital heart disease is actually caused by aberrant noncanonical Notch signaling, instead of the canonical pathway. Understanding noncanonical Notch and Numb signaling will contribute to the study of both cardiogenesis and cancer biology.

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2700 Remington Ave, #320
Baltimore, MD, 21218
Birth Place: Medan, Indonesia

EDRICK SULISTIO

845-842-8032
Esulist1@jhu.edu

Education:

Johns Hopkins University (JHU) Baltimore, MD
Candidate for *Masters of Science in Molecular and Cellular Biology*
Expected graduation in May 2017
Relevant Coursework: Human Genetics, Molecular Biology, Genomes and Development
Thesis title: "The Role of Noncanonical Notch and Numb Signaling During Heart Development"

Johns Hopkins University (JHU) Baltimore, MD
Bachelors of Science in Molecular and Cellular Biology May 2016
Current GPA: 3.76
Dean's List Fall 2014, Spring 2015, Fall 2016
Relevant Coursework: Biochemistry, Genetics, Cell Biology, Developmental Biology, Stem Cells and the Biology of Aging and Disease, Brain Injury and Recovery of Functions, Microbiology, Reproductive Physiology, Animal Cognition

Rockland Community College (RCC) Suffern, NY
Associates in Mathematics/Science May 2014
Overall GPA: 3.975
Dean's List Fall 2012, Spring 2013, Fall 2013
Outstanding Student Service Award Recipient 2013, 2014
Dr. George Krasilovsky Honors Science Scholarship Recipient in May, 2014
Relevant Coursework: General Chemistry, General Biology, Organic Chemistry, Calculus, Physics with calculus

Research Experience:

October '15 – Present **Research Assistant** Baltimore, MD
Johns Hopkins University, Department of Cardiology, Chulan Kwon Laboratory
- Assisted in the study of RNA-binding protein's effect on cardiac development
- Assisted in the study of cardiac organoid development
- Assisted in the study of first heart field and second heart field development
- Techniques utilized included: PCR, QPCR, gel electrophoresis, immunostaining, RNA isolation, cDNA synthesis, and many more

January '15 – May '15 **Research Assistant** Baltimore, MD
Johns Hopkins University, Hilser Laboratory
- Performed point-mutation PCR
- Assisted in the study of glucocorticoid receptor folding process
- Made ampicillin agar for bacterial growth

January '14 – May '14 **Independent Researcher** Suffern, NY
Comparative Neuroanatomy at RCC
- Successfully dissected, retrieved, and compared the brain of frog, cat, pigeon, dogfish, & fetal pig
- Successfully affirmed the evolution of brain anatomical structure

Teaching Experience:

January '17 – May '17 **Teaching Experience, Undergraduate Level Course-General Biology** Baltimore, MD
Department of Biology, Johns Hopkins University
- Graded homework and exams, kept records of the scores using Ms. Excel
- Collaborated with Professors and other TA's on grading policies, improving communication skills

September '16 – December '16 **Teaching Experience, Undergraduate Level Course-Biochemistry** Baltimore, MD
Department of Biology, Johns Hopkins University
- Graded quizzes, homework and exams
- Kept records of scores using Ms. Excel
- Held unofficial office hours to help students understand and solve homework problems

- Collaborated with Professors and other TA's on grading policies, improving communication skills

Volunteer Experience:

February '15 – Present **Volunteer**
Johns Hopkins Hospital Oncology Department Baltimore, MD
- Visit oncology patients to provide companionship by talking, taking them for walks, and etc.
- Help with making education binders for newly diagnosed cancer patients
- Assist in putting up monthly flyers in each patient room
- Help with monthly activities: bingo, movie night, serving dinner to patients/families, and annual concerts

January '15 – Present **Head of Family (team of volunteer)**
Thread Baltimore, MD
- Manage a team of volunteers to help a high school student to achieve academic success
- Provide the student a team of family that will help him solve his personal or academic problems
- Tutored the student in various classes

September '13 – January '14 **Volunteer**
Nyack Hospital Pathology Department Nyack, NY
- Reorganized paperwork requests from doctors to the pathology department
- Examined sample tissues from patients that are sent to the pathology department

January '13 – May '13 **Volunteer**
Nyack Hospital Transportation Department Nyack, NY
- Transported patients from various locations of the hospital
- Provided humor for patients

Leadership Activities:

October '14 – May '16 **Assistant Director/co-Director of Programming**
Inter-Asian Council
JHU Baltimore, MD
- Held events to promote Asian culture
- Raised awareness for Asian American issues

May 18, '14 **Commencement Speaker**
RCC Suffern, NY

September '13 – May '14 **President**
Phi Theta Kappa
RCC Suffern, NY
- Initiated two membership drives for the organization for each semester
- Participated in SUNY Completion Day to encourage students to complete their Associate's Degree

September '13 – May '14 **Member/Group Leader**
Biology Club/Beta Beta Beta
RCC Suffern, NY
- Led a small group of 4 for an experiment to observe the response of planaria to UV rays

September '12–December '13 **Senator**
Student Government Association
RCC Suffern, NY
- Brought food truck to come to the college to provide meals for evening and weekend students
- Raised awareness of the small number of AEDs on campus
- Participated in the blood drive committee, budget and finance committee, student activities board, constitution committee, oversight committee, campus improvement committee, elections committee, publicity committee, and facility committee

- Established, with the approval of the senate, and chaired the Town Hall Meeting Committee
- Advocated students through being a member of Shared Governance Task Force
- Represented students' voice through being a part of the SUNY Chancellor's award committee for Faculty Service in RCC

Special Skills: Fluent in Chinese and Indonesian